ISSN (print) 0093-4666 © 2015. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/130.455

Volume 130, pp. 455–464 *April–June 2015*

Peziza succosella and its ectomycorrhiza associated with *Cedrus deodara* from Himalayan moist temperate forests of Pakistan

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Abstract —Ascomata of *Peziza succosella* and its ectomycorrhiza associated with *Cedrus deodara* were collected from the Himalayan moist temperate forests of Pakistan. The fungal partner in the ectomycorrhiza was identified by sequencing the rDNA internal transcribed spacer region. The morphology of the taxon is also described. Ascomata are characterized by grayish brown deep cupulate apothecia while the ectomycorrhiza is characterized by a thin pseudoparenchymatous mantle with a few emanating elements. *Peziza succosella* and its ectomycorrhiza are an addition to the mycoflora of Pakistan and this is the first report of ectomycorrhizal association of *P. succosella* with Himalayan cedar.

Key words — *Cedrus deodara,* conifers, ITS, *Pezizaceae*, morphotype

Introduction

Peziza Dill. ex Fr. is the largest genus within *Pezizaceae*, with approximately 100 accepted species (Kirk et al. 2008) distributed worldwide. Ten *Peziza* species have been reported from Pakistan: *P. badiofusca* (Boud.) Dennis, *P. cerea* Sowerby, *P. gerardii* Cooke, *P. michelii* (Boud.) Dennis, *P. micropus* Pers., *P. pakistanica* (S. Ahmad) S. Ahmad, *P. repanda* Wahlenb., *P. succosa* Berk., *P. vesiculosa* Bull., and *P. violacea* Pers. (Ahmad et al. 1997, Ashraf & Khalid 2012, Ashraf et al. 2012).

The genus is characterized by epigeous cupulate to discoid sessile to subsessile fleshy ascomata ranging from a few millimetres to more than 10 centimetres in diameter. The majority of the species are considered to be saprotrophs, although ectomycorrhizal species have also been reported (Maia et al. 1996, Hansen et al. 2001, Tedersoo et al. 2006, Ashraf et al. 2012, Jabeen et al. 2012, Jabeen & Khalid 2014). Generally ectomycorrhizae (EcM) of *Pezizales* possess

a thin pseudoparenchymatous mantle, infrequent radiating elements, and no clamp connections (Agerer 1991a, 2001, 2006). Rhizomorphs, if present, do not connect to the ascomata. Identification of EcM of these fungi is difficult using morphological and anatomical methods, but molecular methods have proved to be an efficient identification tool. Combining morphological-anatomical characters with molecular markers is helpful for precise identification of these fungi (Tedersoo et al. 2006, Ashraf & Khalid 2012, Ashraf et al. 2012).

Some pezizalean EcM have already been reported from Pakistan, and most are known to associate with deciduous trees (Ashraf et al. 2012, Jabeen et al. 2012, Jabeen & Khalid 2014). *Peziza succosella* has been reported previously from Israel, Denmark, and Italy (Barseghyan & Wasser 2011, Tedersoo et al. 2006, Osmundson et al. 2013). In Pakistan, the species has been collected from Himalayan moist temperate forests, which extend from Murree hills and Ayubia to Miandam at 1890 to 2500 m above sea level (a.s.l.) between subtropical pine forests and sub-alpine forests dominated by *Abies pindrow* (Royle ex D. Don) Royle, *Cedrus deodara* (Roxb. ex D. Don) G. Don, and *Pinus wallichiana* A.B. Jacks. along with some deciduous trees, notably species of *Populus*, *Quercus*, and *Salix*. *Cedrus deodara* (Himalayan cedar) is an important phytobiont for many EcM fungi (Wang & Qiu 2006, Vaario et al. 2006, Hibbett & Matheny 2009, Hanif et al. 2012). The present work is the first record of the association of *P. succosella* with Himalayan cedar and a new record of this fungus for Pakistan.

Materials & methods

Sampling site

Sampling was carried out during monsoon season from 2010 to 2013. Two sampling sites in Khyber Pakhtunkhwa (KPK) were selected, Khanspur, Ayubia and Sharan, Kaghan Valley. These sites are dominated by moist temperate conifer forests. The areas range from mountains with rugged valleys to undulating and dissected sub-mountain plateaus and flat mountains at 3000–4000 m a.s.l. (Khan 1999). Mean annual temperature of these sites is 10°C (Malik & Sukhera 2012), with temperatures down to 3°C curing December and January and up to 26°C during summer; mean annual rainfall is 1200 mm with 57% humidity (Ahmed et al. 2006). Soil is silt loam to silt clay and non-calcareous to slightly calcareous (Khan 2004). The pH is slightly acidic (≤7) due to high organic content (3–4%) (Irshad & Khan 2012). Among the conifers *Cedrus*, *Picea*, *Pinus*, and *Taxus* dominate, along with some deciduous tree species (Sheikh 1993).

Collection and characterization of ascomata

Specimens were photographed using Nikon D70S digital camera in the field and were carefully removed. At least three individual fruit bodies were collected from each sampling site. Morphological features of fresh specimen were recorded, colors were designated following the Munsell Soil Color Charts (1975), and then the specimens were dried under fan heater. Specimens were sectioned and mounted on slides, and anatomical features were examined a microscope (MX4300H, Meiji Techno Co., Ltd.,

Japan). Structural components of the excipulum were studied in free hand sections and hymenial elements were studied by tearing apart a piece of hymenium using a needle. Measurements were recorded using Carl Zeiss Jena ocular micrometer and line drawing were made using Leitz Wetzlar Camera Lucida. The abbreviation n/m/p means n ascospores were measured from m ascomata and p collections. Voucher specimens were deposited in the Herbarium, Botany Department, University of the Punjab, Lahore, Pakistan (LAH).

Collection, isolation, and characterization of EcM

Sampling was carried out very carefully to make sure that the fine roots in the soil block belonged to the same tree. A 15-cm^3 soil block was dug with a shovel a few centimetres from the tree trunk. Five soil cores were taken from each sampling site. In the laboratory, each soil core was soaked in water for a few hours to loosen the soil particles and then put on a 2 mm sieve under running water to separate the roots from the soil. The EcM were carefully sorted into morphotypes under incandescent light and then under a stereomicroscope (EMZ-5TR, Meiji Techno Co., Ltd., Japan). The morphotypes were cleaned under the stereomicroscope using a fine brush. Morphologically identical morphotypes were kept in McCartney bottles in distilled water for future analysis. Replicates of these morphotypes were kept at 8°C in Eppendorf tubes containing 2% CTAB buffer for molecular analysis. Morphological characters (e.g., color, size, ramification, presence or absence of radiating elements) were noted under the stereomicroscope. Mantle layers (inner and outer) and radiating hyphae were mounted in trypan blue stain (Agerer 1991b). Hyphae were measured using an ocular micrometer and drawn using a camera lucida. A voucher specimen was deposited in LAH Herbarium.

DNA extraction, amplification and sequencing

For molecular analysis, up to 2 mg of ascomatal tissue was placed in one Eppendorf tube and 2–4 ectomycorrhizal root tips were placed in a separate Eppendorf tube. DNA was extracted using modified CTAB method (Bruns 1995). Internal Transcribed Spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) were amplified using universal primer pair (ITS1F: 5′-CTTGGTCATTTAGAGGAAGT-3′ and ITS4: 5′- TCCTCCGCTTATTGATATGC-3′) following Gardes & Bruns (1993). The products were sent to Macrogen Inc. (Korea) for sequencing.

Molecular phylogenetic analysis

Consensus sequences were generated from the obtained sequences and then BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). Sequences with the closest matches were selected from GenBank to reconstruct a phylogeny. Sequences that showed less query cover and negative E value were not included. Published sequences of the closest relatives of the species were also included in the final data set, and *Sarcosphaera coronaria* (Jacq.) J. Schröt. was chosen as outgroup (Tedersoo et al. 2006). ClustalW was used to align sequences in BioEdit software. The sequences were trimmed with the conserved motifs 5 -(…GAT) CATTA… and…GACCT (CAAA…)-3 , and the alignment portions between them was used to reconstruct phylogeny. Maximum likelihood (ML) analysis was performed using Jukes-Cantor model in MEGA6 software to test the phylogeny at 1000 bootstraps. Percentage identity and divergence in nrDNA-ITS was analysed using

MegAlign (DNAStar). Sequences generated in this study were submitted to GenBank (KM199728, KM199729).

Taxonomy

Peziza succosella (Le Gal & Romagn.) M.M. Moser ex Aviz.-Hersh. & Nemlich, Israel J. Bot. 23(3): 156 (1974). Fig. 1

Apothecia deeply cupulate, sessile to sub-sessile, circular, centrally attached, initially circular, irregular at older stage, medium-sized (1.5–2.5 cm). Hymenium smooth, olivaceous grey (10YR6/2), outer surface smooth, pale grey (5Y7/2). Asci cylindrical, operculate, unitunicate, 8-spored, uniseriate, strongly amyloid at apex, slightly narrowing towards base, 253–290 × 15–17 µm. Ascospores [50/4/2] ellipsoid, uniguttulate, light brown, ornamented, (15–)16–18 × 8–11 µm (16.93 ± 0.815 × 9.66 ± 0.59; Qm = 1.75 ± 0.12), ornamentation longitudinal warts to partially reticulate, 1.2–2.0 µm high warts. Paraphyses slender, septate near base, same diam. throughout length, same length as asci, ≤4.3–5.0 µm wide. Excipulum ectal textura globosa to textura angularis, slightly brown cells, 13–23 × 15–20 µm, globose cells of ectal smaller as compared to inner angular cells, ectal cells ending in hyphoid extensions that appear septate, hyaline, blunt ended, 8–11 µm wide.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa**, Ayubia, Khanspur Village, 2575 m a.s.l., in Himalayan moist temperate forests, on ground, damp soil, 21 August 2010, Tayiba Ashraf; TA-135 (LAH210810); Kaghan Valley, Sharan, 2011 m a.s.l., in Himalayan moist temperate forests, 14 August 2011, Tayiba Ashraf, TA-221(LAH140811; GenBank KM199729).

Morphological characterization of EcM

Fig. 2

Ectomycorrhizal system dichotomous to coralloid, explored at upper layer of soil, ≤4 mm long, axis ≤400 µm in diameter, un-ramified ends straight, not inflated and cylindrical, ≤400 µm in diameter, width of tip base and apex 300 µm, grayish brown to dark brown or black at maturity. Mantle grayish (5Y7/2) to transparent, distinct, smooth surface, luster matte; host tissue visible under the mantle surface. Rhizomorphs absent. Emanating hyphae transparent, infrequent, sometimes frequent at few tips, straight, cylindrical rarely branched.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa**, Ayubia, Khanspur Helipad, 2575 m a.s.l., in Himalayan moist temperate forests, mycorrhiza associated with *Cedrus deodara,* 21 September 2013, Sana Jabeen; SA116; HP-5 (LAH-EM1-2013; GenBank KM199728).

Anatomical characteristics of mantle in plan view

Mantle pseudoparenchymatous in all layers, non-gelatinous, angular cells with blunt corners. Outer mantle layer pseudoparenchymatous, cells angular, (mantle type K, Agerer 1987–2002; Agerer & Rambold 1998), cells

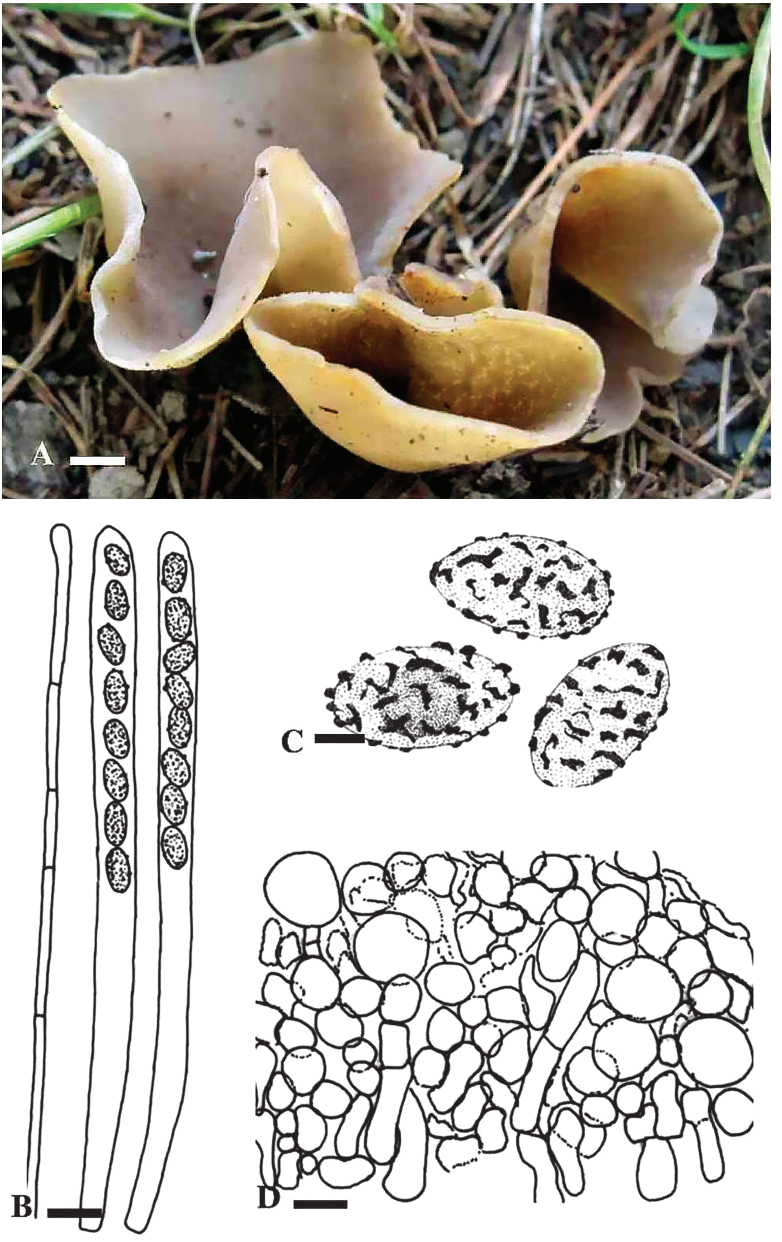

Fig. 1. *Peziza succosella* (LAH140811). A. Apothecia. B. Asci with ascospores and paraphyses. C. Ornamented ascospores. D. Part of excipulum. Scale bars: A = 1.5 cm; B, D = 25 µm; C = 5 µm.

membranaceously and plasmatically transparent, 11.56 × 10.01 ± 9.05 µm, matrix clear. Inner mantle layer pseudoparenchymatous, angular cells with blunt corners, (mantle type K, Agerer 1987–2002; Agerer & Rambold 1998), cells membranaceously and plasmatically transparent, 12.46 ×10.05 ± 10.07 µm, cell contents clear.

Anatomical characteristics of emanating elements

Emanating hyphae elongated, straight, cylindrical, up to 4.74 µm in diameter, wall up to 0.70 µm thick, surface smooth, hyphae septate, septa frequent, clamps absent, septa 32.70 µm apart, contents clear, ramification rather frequent, Y-shaped, anastomosing; cystidia not observed. Rhizomorphs absent.

Molecular Phylogenetic Characterization

Sequences of the ITS region of nrDNA of fruit body and EcM were BLAST searched at NCBI. BLAST search revealed that the subject sequences were 99% identical with *P. succosella* (JF908535) and a specimen identified as *P. subumbrina* Boud. (JF908552) from Italy with 100% and 96% query cover respectively, and 0.0 E value. Both Pakistani sequences — TA-221 (fruit body) and SA116 (EcM) — clustered with *P. succosella* from Italy and Denmark (DQ200841) with 99% boot strap support; these form a sister clade with *P. infossa* and *P. succosa* in the phylogenetic tree with 92% bootstrap support (Fig. 3). Both sequences showed a 99.8% genetic identity with *P. succosella* (JF908535), a 97.2% with the questionably identified Italian specimen of *P. subumbrina* (JF908552), and a 92.7 % genetic identity with *P. succosella* (DQ200841) from Denmark with 0 value of genetic divergence. The low value of genetic identity of *P. succosella* (DQ200841) is due to low query cover (89%) in BLAST.

Discussion

Pezizalean species constitute an important part of mycobiont diversity in EcM fungal communities (Tedersoo et al. 2006). Many EcM fungal taxa belonging to this group have been reported from Himalayan moist temperate forests of Pakistan (Ashraf et al. 2012, Hanif et al. 2012). EcM morphology of pezizalean taxa is little studied. Previously published morphological and anatomical descriptions of pezizalean EcM cover only a few genera. EcM of *P. succosella* are grayish brown and showed dichotomous to coralloid type of ramification, presence of a pseudoparenchymatous thin mantle layer with few emanating hyphae, and lack of clamped septa. These characters are distinct from the previously reported EcM of *P. michelii* having yellow green to olive green morphotypes (Tedersoo et al. 2006) and cream white with brown to black older tips (Ashraf et al. 2012). Cells of the mantle layer of *P. michelii* differ greatly in size and shape from each other while the cells of mantle layer of *P. succosella* showed comparatively less variability in size and shape among

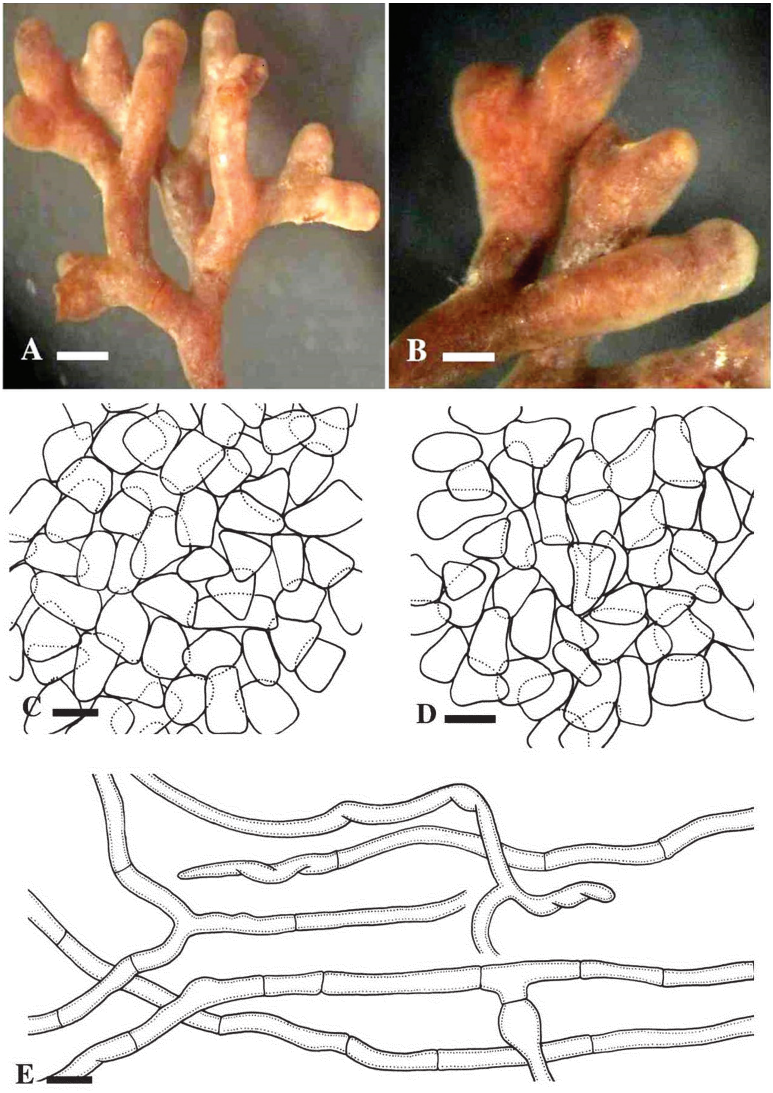

Fig. 2. EcM of *Peziza succosella*. A. Ectomycorrhizal system. B. Root tip morphology. C. Inner mantle layer. D. Outer mantle layer. E. Emanating hyphae. Scale bars: A = 0.5 mm; B = 0.25 mm; C–E = 10 μm.

themselves. Sequences of the fruit body and EcM along with morphological descriptions were found helpful in identification. ITS phylogenetic analysis revealed that the sequences derived from ascocarp and ectomycorrhizal tissue

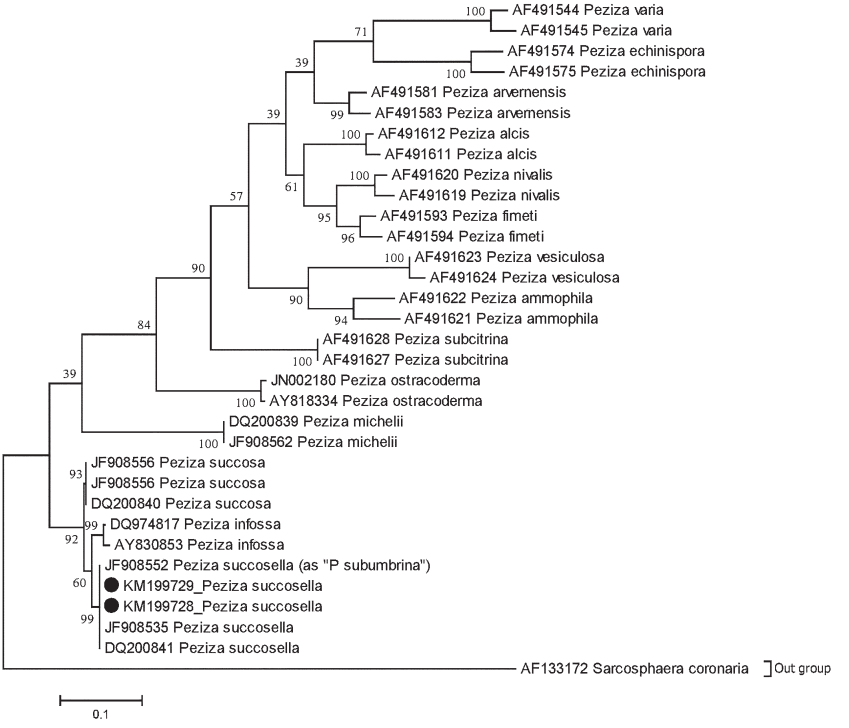

Fig. 3. Molecular phylogenetic analysis of *Peziza succosella* and related species. New sequences generated from Pakistan are marked with ●. Genbank accession numbers of all taxa are given. The percentage of trees in which the associated taxa clustered together at 1000 bootstraps is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 33 nucleotide sequences. There were a total of 885 positions in the final dataset.

clustered with *P. succosella* from Italy and Denmark with very strong bootstrap value. Its ectomycorrhiza has previously been undescribed. The molecular distinction among specimens from Pakistan and other countries is minimal, placing the ascocarps and EcM of *Peziza succosella* in the same species, despite the large geographic distance. This indicated a wide species distribution and a circum-Mediterranean distribution pattern.

Acknowledgments

Sincere thanks to Dr. Tine Grebenc (Slovenian Forestry Institute, Ljubljana) for his help in improving the manuscript. We are also thankful to Dr. T.K. Arun Kumar

(The Zamorin's Guruvayurappan College, India) and Prof. Donald H. Pfister (Harvard University Herbaria, Cambridge, MA, USA) for acting as pre submission reviewers. Thanks to Mr. Hassan Ayub for his help in collecting EcM. The work is financially supported by Higher Education Commission (HEC)-Pakistan under Indigenous PhD Fellowships for 5000 Scholars, HEC (Phase-II).

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